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Alan [GB/NO]; Nycomed Imaging AS, Nycoveien 1-2,
P.O. Box 4220 Nydalen, N-N-0401 Oslo (NO).

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(71) Applicant (*for all designated States except US*): NY-COMED IMAGING AS [NO/NO]; Intellectual Property Dept., Nycoveien 1-2, P.O. Box 4220 Nydalen, N-N-0401 Oslo (NO).

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(72) Inventor; and

(75) Inventor/Applicant (*for US only*): CUTHBERTSON,

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(54) Title: PEPTIDE-BASED COMPOUNDS

(57) Abstract: This invention relates to new peptide-based compounds and their use in therapeutically effective treatments as well as for diagnostic imaging techniques. More specifically the invention relates to the use of such peptide-based compounds used as targeting vectors that bind to receptors associated with angiogenesis, in particular the avb3 integrin receptor. Such contrast agents may thus be used for diagnosis of for example malignant diseases, heart diseases, inflammation-related diseases, rheumatoid arthritis and Kaposi's sarcoma. Moreover such compounds may also be used in therapeutic treatment of these diseases.

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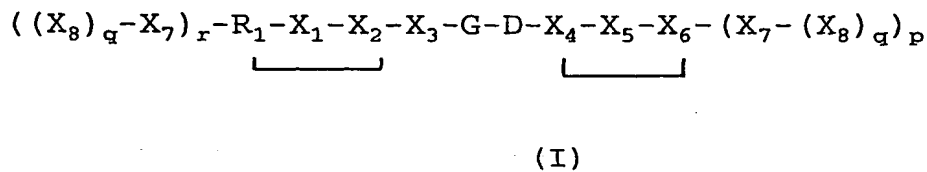
Cyclic RGD peptides where the RGD moiety is constrained by bridging across the ends of the tripeptide sequence have also been described in WO98/54347 and WO95/14714. Peptides derived from in vivo biopanning (WO97/10507) have been used for a variety of targeting applications. The sequence CDCRGDCFC (RGD-4C), with unidentified bridge positions, has been used to target drugs such as doxorubicin (WO98/10795), nucleic acids and adenoviruses to cells (see WO99/40214, WO99/39734, WO98/54347, WO98/54346, US 5846782).

The efficient targeting and imaging of integrin receptors associated with angiogenesis in vivo demands a selective, high affinity RGD based vector which is chemically robust and stable. Furthermore, the route of excretion is an important factor when designing imaging agents in order to reduce problems with background. These stringent conditions are met by the structures containing discrete bridges described in the present invention.

Viewed from one aspect the invention provides new peptide-based compounds as defined by formula I. These compounds have utility as vectors with affinity for the integrin $\alpha v \beta 3$ and comprising a linear RGD sequence flanked by two discrete bridges where one or both of the bridges is a disulphide bridge. Such vectors have now shown unexpected activity by improved binding/efficacy compared to known linear RGD peptides. These new peptide-based compounds may be used in therapeutically effective treatments as well as for imaging purposes.

Formula I thus defines peptide-based compounds used as vectors (V) having affinity for the integrin $\alpha v \beta 3$. However, depending on the definitions of R_1 and X_{1-8} , formula I also includes compounds of the formula "V-L-R" where

V is the vector, L is a linker moiety or a bond, and R is a detectable moiety (reporter), e.g. detectable in an imaging procedure, such as *in vivo* imaging of the human or vascularized non-human animal body (e.g. mammalian, avian or reptilian body), wherein said compound is characterised by the general formula (I)



wherein

$r = 0$ or 1

$p = 0$ or 1

and $r+p = 1$

when $r = 0$ then R_1 is $-(CH_2)_n-CO-$ or $-(CH_2)_n-C_6H_4-CO-$
where $n=1,2,3,4$ or 5 ,

when $r = 1$ then R_1 is or one or more bridge-forming amino acids, preferable cysteine and preferably the bridge between R_1 and X_2 contains a thioether or a disulphide bond,

and when $r=1$ (and thus $p=0$) then R_1 is particularly preferably cysteine and forms a disulphide bridge with X_2 ,

X_1 = a bond or 1, 2, 3, 4 or 5 amino acids, or an amino acid derivatized with a carbohydrate moiety, or an amino acid functionalised with a spacer or linker and/or a chelate binding or capable of binding a reporter suitable for *in vivo* imaging, preferably a metal radionuclide,

preferably X_1 is aspartic acid, tyrosine, tyrosine-aspartic acid or lysine,

X_2 and X_4 are independently cysteine, homocysteine or other amino acids capable of forming a cyclising bond such as aspartic acid and lysine,

X_3 is arginine, N-methylarginine or an arginine mimetic,

X_5 is a hydrophobic amino acid, preferably phenylalanine, tyrosine, iodotyrosine (most preferably 3-iodo-tyrosine), diiodotyrosine or naphthylalanine,

X_6 is an amino acid capable of forming a cyclising bond, preferable cysteine or homocysteine,

X_7 is a bond or 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids, preferably glycine, or a spacer or linker, optionally allowing for labelling with multiple chelates as defined by X_8 , and optionally comprising one or more ethylene glycol units or any other spacer component, and

X_8 is a chelate binding to, or capable of binding a metal radionuclide or any other reporter suitable for in vivo imaging, or is $-NH_2$ or is absent,

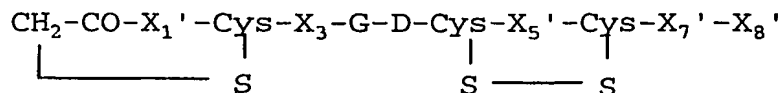
q is 0, 1, 2, 3, 4, 5, 6, 7 or 8, and

one of the bridges (between R_1 and X_2 or between X_4 and X_6) comprises a disulphide bond.

The vector components of the vector-chelate conjugates described herein have in some aspects of the invention no free amino- or carboxy-termini. Such termini introduce into these compounds a significant increase in resistance against enzymatic degradation and as a result

they have an increased *in vivo* stability as compared to many known free peptides.

The invention relates preferably to a compound of formula (I), further defined by formula (II)



(II) where Cys= Cysteine

wherein

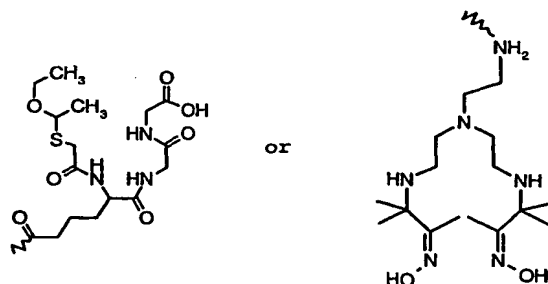
X₁' is 1, 2 or 3 amino-acids, most preferably aspartic acid, tyrosine, tyrosine-aspartic acid, lysine or acetyl-lysine,

X₃ is as defined in formula I;

X₅' is phenylalanine, tyrosine, 3-iodo-tyrosine or naphthylalanine;

X₇' is a bond, glycine, or a O-bis(aminoethyl)ethylene glycol spacer, preferably X₇ is glycine; and

X₈' is a chelate binding to a metal radionuclide wherein the structure of the chelate is



or any other N₃S or bis-oxime type of these chelates, and

one bridge comprises a thio bond and the other bridge comprises a disulphide bond as shown in formula II.

In particularly preferred embodiments of the compounds of general formula (II), X_1 is N-methyl-arginine and/or X_2 is naphthylalanine.

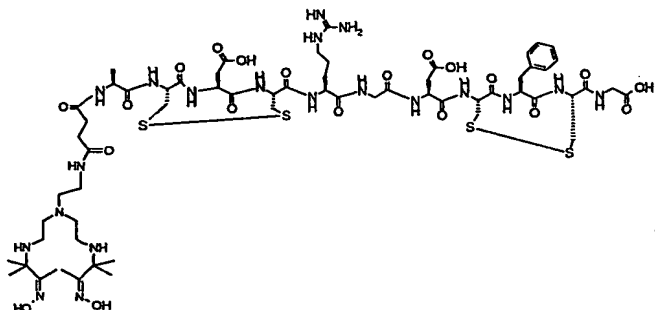
The reporter, R, may be attached to V (via L) at any suitable point within X_1 and/or X_2 . Preferably, the point of attachment is chosen such that the biological activity of V or the binding affinity of V for its target is not substantially or not significantly reduced (in comparison with the biological activity of V or the binding affinity of V without R). Most preferably, R is attached to V via X_1 and/or X_2 .

As used herein the term 'amino acid' refers in its broadest sense to proteogenic L-amino acids, D-amino acids, chemically modified amino acids, N-methyl, C-methyl and amino acid side-chain mimetics and unnatural amino acids such as naphthylalanine.

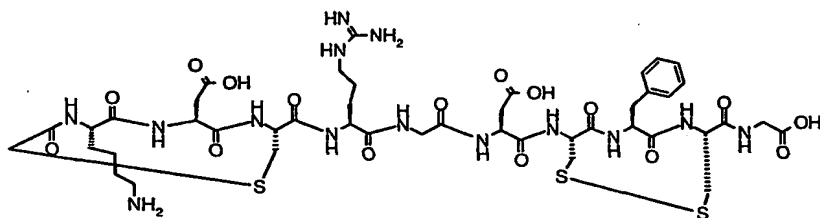
The term 'cyclising bond' refers to any combination of amino acids (or with amino acid and $-(CH_2)_n-CO-$ or $-(CH_2)_n-C_6H_4-CO-$) with functional groups which allows for the introduction of a bridge. Some preferred examples are disulphides, disulphide mimetics such as the $-(CH_2)_4-$ carba bridge, thioacetal, thioether bridges (cystathione or lanthionine) and bridges containing esters and ethers.

Some preferred embodiments of the compounds of formula (I) are illustrated by Compounds 1-5 below:

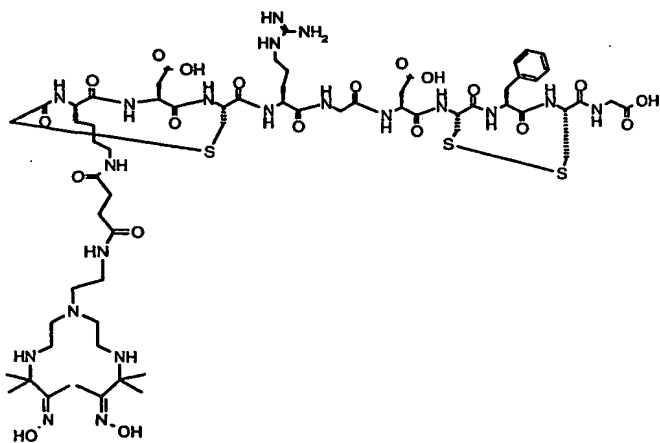
Compound 1:



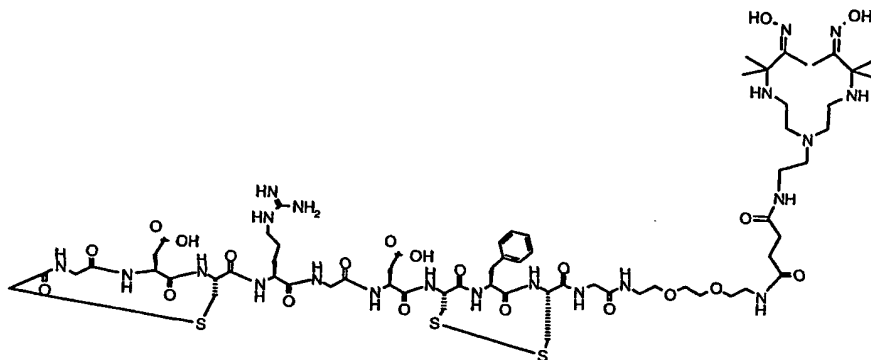
Compound 2:- Vector



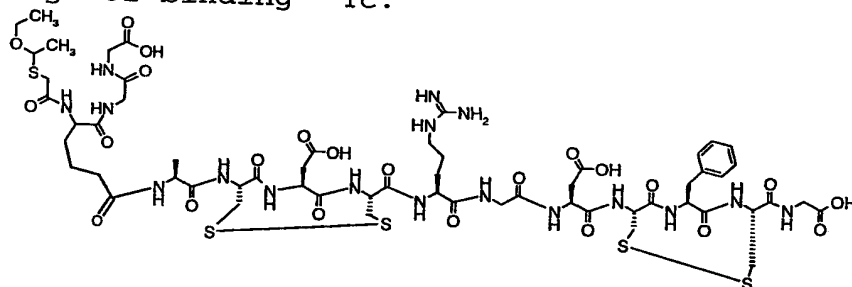
Compound 3: Example of a V-L-R compound of formula I



Compound 4: Example of a compound of formula II



Compound 5: Example of a compound of formula capable e.g. of binding ^{99m}Tc .



In most cases, it is preferred that the amino-acids in the vector V are in the L-form. However, in some embodiments of the invention one, two, three or more of the amino-acids in the vector V are preferably in the D-form. The inclusion of such D-form amino-acids can have a significant effect on the serum stability of the vector. Reference is particularly made in this regard to vectors having D-tyrosine at position X_1 .

The invention also provides a pharmaceutical composition comprising an effective amount (e.g. an amount effective to enhance image contrast in *in vivo* imaging and/or for therapeutic treatment) of a compound of general formula (I) or an acid addition salt thereof, together with one

or more pharmaceutically acceptable adjuvants, excipients or diluents.

As mentioned above, the compounds of formula I may comprise vector, linker and reporter moieties. A linker moiety may serve to link one vector to one reporter; alternatively it may link together more than one vector and/or more than one reporter. Likewise a reporter or a vector may be linked to more than one linker. Use in this way of a plurality of reporters (e.g. several linker-reporter moieties attached to one vector or several reporters attached to one linker itself attached to one vector) may enable the detectability of the contrast agent to be increased (e.g. by increasing its radioopacity, echogenicity or relaxivity) or may enable it to be detected in more than one imaging modality. Use in this way of a plurality of vectors may increase the targeting efficiency of the contrast agent or may make the contrast agent able to target more than one site, e.g. different receptors for an agent which has receptor heterogeneity.

Linker

A wide variety of linkers can be used, including biodegradable linkers and biopolymers.

The linker component of the contrast agent is at its simplest a bond between the vector and reporter moieties. More generally however the linker will provide a mono- or multi-molecular skeleton covalently or non-covalently linking one or more vectors to one or more reporters, e.g. a linear, cyclic, branched or reticulate molecular skeleton, or a molecular aggregate, with in-built or pendant groups which bind covalently or non-covalently, e.g. coordinatively, with the vector and reporter moieties or which encapsulate, entrap or anchor such moieties.

Thus linking of a reporter unit to a desired vector may be achieved by covalent or non-covalent means, usually involving interaction with one or more functional groups located on the reporter and/or vector. Examples of chemically reactive functional groups which may be employed for this purpose include amino, hydroxyl, sulfhydryl, carboxyl, and carbonyl groups, as well as carbohydrate groups, vicinal diols, thioethers, 2-aminoalcohols, 2-aminothiols, guanidinyll, imidazolyl and phenolic groups.

It will be appreciated that functional groups in the reporter and/or vector may if desired be converted to other functional groups prior to reaction, e.g. to confer additional reactivity or selectivity.

Vector-reporter coupling may also be effected using enzymes as zero-length crosslinking agents; thus, for example, transglutaminase, peroxidase and xanthine oxidase have been used to produce crosslinked products. Reverse proteolysis may also be used for crosslinking through amide bond formation.

Non-covalent vector-reporter coupling may, for example, be effected by electrostatic charge interactions, through chelation in the form of stable metal complexes or through high affinity binding interaction.

A vector which is coupled to a peptide, lipo-oligosaccharide or lipopeptide linker which contains a element capable of mediating membrane insertion may also be useful.

Coupling may also be effected using avidin or streptavidin, which have four high affinity binding sites for biotin. Avidin may therefore be used to

conjugate vector to reporter if both vector and reporter are biotinylated.

So-called zero-length linking agents, which induce direct covalent joining of two reactive chemical groups without introducing additional linking material may, if desired, be used in accordance with the invention.

Most commonly, however, the linking agent will comprise two or more reactive moieties, e.g. as described above, connected by a spacer element. The presence of such a spacer permits bifunctional linkers to react with specific functional groups within a molecule or between two different molecules, resulting in a bond between these two components and introducing extrinsic linker-derived material into the reporter-vector conjugate.

The nature of extrinsic material introduced by the linking agent may have a critical bearing on the targeting ability, pharmacokinetics and general stability of the ultimate product. Thus it may be desirable to introduce labile linkages, e.g. containing spacer arms which are biodegradable or chemically sensitive or which incorporate enzymatic cleavage sites. Alternatively the spacer may include polymeric components, e.g. to act as surfactants and enhance the stability of the agent. The spacer may also contain reactive moieties, e.g. as described above to enhance surface crosslinking.

Spacer elements may also comprise macromolecular structures such as dextran and poly(ethyleneglycols), usually referred to as PEGs. In addition to spacer elements, PEGs may also be used to modify the *in vivo* characteristics of the vectors.

The major mechanism for uptake of particles by the cells of the reticuloendothelial system (RES) is opsonisation by plasma proteins in blood; these mark foreign particles which are then taken up by the RES. The biological properties of PEG spacer elements used in accordance with the invention may serve to increase the circulation time of the agent in a similar manner to that observed for PEGylated liposomes. Increased coupling efficiency to areas of interest may also be achieved using antibodies bound to the termini of PEG spacers.

Other representative spacer elements include structural-type polysaccharides, storage-type polysaccharides, polyamino acids and methyl and ethyl esters thereof, and polypeptides, oligosaccharides and oligonucleotides, which may or may not contain enzyme cleavage sites.

Preferred linking groups are derived from vector reactive groups selected from but not limited to:-

a group that will react directly with carboxy, aldehyde, amine (NHR), alcohols, sulfhydryl groups, activated methylenes and the like, on the vector, for example, active halogen containing groups,

a group that can react readily with modified vector molecules containing a vector reactive group, i.e., vectors containing a reactive group modified to contain reactive groups, for example, by oxidation of the vector to an aldehyde or a carboxylic acid, and

a group that can be linked to the vector containing a reactive group, or to the modified vector as noted above by use of a crosslinking agent.

Preferred useful linking groups are derived from various heterobifunctional cross-linking reagents such as those listed in the Pierce Chemical Company Immunotechnology Catalog - Protein Modification Section, (1995 and 1996).

In addition to the foregoing description, the linking groups, in whole or in part, can also be comprised of and derived from complementary sequences of nucleotides and residues of nucleotides, both naturally occurring and modified, preferably non-self-associating oligonucleotide sequences.

Linking agents used in accordance with the invention will in general bring about linking of vector to reporter or reporter to reporter with some degree of specificity, and may also be used to attach one or more therapeutically active agents.

Further examples of the linkers which may be used in the context of the current application are given on pages 32-54 of WO98/47541 and the disclosures made on these pages are incorporated herein by reference in their entirety. It is hereby asserted that each and every linker or part thereof disclosed on the aforementioned pages is considered to be part of the description of the invention contained in this application.

Reporter

The reporter moieties in the contrast agents of the invention may be any moiety capable of detection either directly or indirectly in an *in vivo* diagnostic imaging procedure, e.g. moieties which emit or may be caused to emit detectable radiation (e.g. by radioactive decay, fluorescence excitation, spin resonance excitation, etc.), moieties which affect local electromagnetic fields (e.g. paramagnetic, superparamagnetic, ferrimagnetic or ferromagnetic species), moieties which

absorb or scatter radiation energy (e.g. chromophores and fluorophores), particles (including liquid containing vesicles), heavy elements and compounds thereof, and moieties which generate a detectable substance, etc..

A very wide range of materials detectable by diagnostic imaging modalities is known from the art and the reporter will be selected according to the imaging modality to be used. Thus for example for ultrasound imaging an echogenic material, or a material capable of generating an echogenic material will normally be selected. The vectors may be coupled via a linker to a suitable lipid reporter/carrier for incorporation into a gas-filled microbubble. Such microbubbles may be used for targeting ultrasound imaging.

For X-ray imaging the reporter will generally be or contain a heavy atom (e.g. of atomic weight 38 or above). For MR imaging the reporter will either be a non zero nuclear spin isotope (such as ^{19}F) or a material having unpaired electron spins and hence paramagnetic, superparamagnetic, ferrimagnetic or ferromagnetic properties. For light imaging the reporter will be a light scatterer (e.g. a coloured or uncoloured particle), a light absorber or a light emitter. For magnetometric imaging the reporter will have detectable magnetic properties; for electrical impedance imaging the reporter will affect electrical impedance. For scintigraphy, SPECT, PET, etc., the reporter will be a radionuclide.

Examples of suitable reporters are widely known from the diagnostic imaging literature, e.g. magnetic iron oxide particles, X-ray contrast agent containing vesicles, chelated paramagnetic metals (such as Gd, Dy, Mn, Fe etc.). See for example US-A-4647447,

PCT/GB97/00067, US-A-4863715, US-A-4770183, WO96/09840, WO85/02772, WO92/17212, PCT/GB97/00459, EP-A-554213, US-A-5228446, WO91/15243, WO93/05818, WO96/23524, WO96/17628, US-A-5387080, WO95/26205, GB9624918.0, etc. See also WO 98/47541 (pages 63-66 and 70-86).

Particularly preferred as reporters are: chelated paramagnetic metal ions such as Gd, Dy, Fe, and Mn, especially when chelated by macrocyclic chelant groups.

Stated generally, the reporter may be (1) a chelatable metal or polyatomic metal-containing ion (i.e. TcO, etc), where the metal is a high atomic number metal (e.g. atomic number greater than 37), a paramagnetic species (e.g. a transition metal or lanthanide), or a radioactive isotope, (2) a covalently bound non-metal species which is an unpaired electron site (e.g. an oxygen or carbon in a persistent free radical), a high atomic number non-metal, or a radioisotope, (3) a polyatomic cluster or crystal containing high atomic number atoms, displaying cooperative magnetic behaviour (e.g. superparamagnetism, ferrimagnetism or ferromagnetism) or containing radionuclides, (4) a chromophore (by which term species which are fluorescent or phosphorescent are included), e.g. an inorganic or organic structure, particularly a complexed metal ion or an organic group having an extensive delocalized electron system, or (5) a structure or group having electrical impedance varying characteristics, e.g. by virtue of an extensive delocalized electron system.

Examples of particular preferred reporter groups are described in more detail below.

Chelated metal reporters: metal radionuclides, paramagnetic metal ions, fluorescent metal ions, heavy metal ions and cluster ions.

Preferred metal radionuclides include ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{47}Sc , ^{67}Ga , ^{51}Cr , $^{177\text{m}}\text{Sn}$, ^{67}Cu , ^{167}Tm , ^{97}Ru , ^{188}Re , ^{177}Lu , ^{199}Au , ^{203}Pb and ^{141}Ce .

Preferred paramagnetic metal ions include ions of transition and lanthanide metals (e.g. metals having atomic numbers of 6 to 9, 21-29, 42, 43, 44, or 57-71), in particular ions of Cr, V, Mn, Fe, Co, Ni, Cu, La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu, especially of Mn, Cr, Fe, Gd and Dy, more especially Gd.

The metal ions are desirably chelated by chelant groups on the linker moiety or in or on a particle, (e.g. a vesicle or a porous or non-porous inorganic or organic solid), in particular linear, macrocyclic, terpyridine and N_2S_2 chelants, such as for example DTPA, DTPA-BMA, EDTA, D03A and TMT. Further examples of suitable chelant groups are disclosed in US-A-4647447, WO89/00557, US-A-5367080, US-A-5364613, etc..

The linker moiety or the particle may contain one or more such chelant groups, if desired metallated by more than one metal species (e.g. so as to provide reporters detectable in different imaging modalities).

Other suitable residues of chelating agents comprise proteins modified for the chelation of metals such as technetium and rhenium as described in US Patent No. 5078985, the disclosure of which is hereby incorporated by reference.

Methods for metallating any chelating agents present are within the level of skill in the art. Metals can be incorporated into a chelant moiety by any one of three general methods: direct incorporation, template synthesis and/or transmetallation. Direct incorporation is preferred.

Thus it is desirable that the metal ion be easily complexed to the chelating agent, for example, by merely exposing or mixing an aqueous solution of the chelating agent-containing moiety with a metal salt in an aqueous solution preferably having a pH in the range of about 4 to about 11. The salt can be any salt, but preferably the salt is a water soluble salt of the metal such as a halogen salt, and more preferably such salts are selected so as not to interfere with the binding of the metal ion with the chelating agent. The chelating agent-containing moiety is preferably in aqueous solution at a pH of between about 5 and about 9, more preferably between pH about 6 to about 8. The chelating agent-containing moiety can be mixed with buffer salts such as citrate, acetate, phosphate and borate to produce the optimum pH. Preferably, the buffer salts are selected so as not to interfere with the subsequent binding of the metal ion to the chelating agent.

In diagnostic imaging, the vector-linker-reporter (VLR) construct preferably contains a ratio of metal radionuclide ion to chelating agent that is effective in such diagnostic imaging applications. In preferred embodiments, the mole ratio of metal ion per chelating agent is from about 1:1,000 to about 1:1.

In radiotherapeutic applications, the VLR preferably contains a ratio of metal radionuclide ion to chelating agent that is effective in such therapeutic

applications. In preferred embodiments, the mole ratio of metal ion per chelating agent is from about 1:100 to about 1:1. The radionuclide can be selected, for example, from radioisotopes of Sc, Fe, Pb, Ga, Y, Bi, Mn, Cu, Cr, Zn, Ge, Mo, Ru, Sn, Sr, Sm, Lu, Sb, W, Re, Po, Ta and Tl. Preferred radionuclides include ^{44}Sc , ^{64}Cu , ^{67}Cu , ^{212}Pb , ^{68}Ga , ^{90}Y , ^{153}Sm , ^{212}Bi , ^{186}Re and ^{188}Re . Of these, especially preferred is ^{90}Y . These radioisotopes can be atomic or preferably ionic.

The following isotopes or isotope pairs can be used for both imaging and therapy without having to change the radiolabeling methodology or chelator: $^{47}\text{Sc}_{21}$; $^{141}\text{Ce}_{58}$; $^{188}\text{Re}_{75}$; $^{177}\text{Lu}_{71}$; $^{199}\text{Au}_{79}$; $^{47}\text{Sc}_{21}$; $^{131}\text{I}_{53}$; $^{67}\text{Cu}_{29}$; $^{131}\text{I}_{53}$ and $^{123}\text{I}_{53}$; $^{188}\text{Re}_{75}$ and $^{99\text{m}}\text{Tc}_{43}$; $^{90}\text{Y}_{39}$ and $^{87}\text{Y}_{39}$; $^{47}\text{Sc}_{21}$ and $^{44}\text{Sc}_{21}$; $^{90}\text{Y}_{39}$ and $^{123}\text{I}_{53}$; $^{146}\text{Sm}_{62}$ and $^{153}\text{Sm}_{62}$; and $^{90}\text{Y}_{39}$ and $^{111}\text{In}_{49}$.

The linker moiety may also allow for coupling to a plurality of chelant groups. The chelant moieties within such a polychelant linker may be attached via backbone functionalization of the chelant or by utilization of one or more of the metal co-ordinating groups of the chelant or by amide or ether bond formation between acid chelant and an amine or hydroxyl carrying linker backbone, e.g. as in polylysine-polyDTPA, polylysine-polyDOTA and in the so-called magnifier polychelants, of PCT/EP96/00565. Such polychelant linkers may be conjugated to one or more vector groups either directly (e.g. utilizing amine, acid or hydroxyl groups in the polychelant linker) or via a bifunctional linker compound as discussed above for monochelant linkers.

Where the chelated species is carried by a particulate (or molecular aggregate, e.g. vesicular) linker, the chelate may for example be an unattached mono or polychelate (such as Gd DTPA-BMA or Gd HP-DO3A)

enclosed within the particle or it may be a mono or polychelate conjugated to the particle either by covalent bonding or by interaction of an anchor group (e.g. a lipophilic group) on the mono/polychelate with the membrane of a vesicle (see for example PCT/GB95/02378).

Preferred non-metal atomic reporters include radioisotopes such as ^{123}I and ^{131}I as well as non zero nuclear spin atoms such as ^{19}F , and heavy atoms such as I.

Such reporters, preferably a plurality thereof, e.g. 2 to 200, may be covalently bonded to a linker backbone, either directly using conventional chemical synthesis techniques or via a supporting group, e.g. a triiodophenyl group.

In an embodiment of this invention, the use of radioisotopes of iodine is specifically contemplated. For example, if the vector or linker is comprised of substituents that can be chemically substituted by iodine in a covalent bond forming reaction, such as, for example, substituents containing hydroxyphenyl functionality, such substituents can be labeled by methods well known in the art with a radioisotope of iodine. The iodine species can be used in therapeutic and diagnostic imaging applications. While, at the same time, a metal in a chelating agent on the same vector-linker can also be used in either therapeutic or diagnostic imaging applications.

As with the metal chelants discussed above, such metal atomic reporters may be linked to the linker or carried in or on a particulate linker, e.g. in a vesicle (see WO95/26205 and GB 9624918.0).

Linkers of the type described above in connection with the metal reporters may be used for non-metal atomic reporters with the non-metal atomic reporter or groups carrying such reporters taking the place of some or all of the chelant groups.

Preferably the V-L-R agents of the invention will have the receptor targetting vectors coupled directly or indirectly to a reporter, e.g. with covalently bound iodine radioisotopes, with metal chelates attached directly or via an organic linker group or coupled to a particulate reporter or linker-reporter, e.g. a superparamagnetic crystals (optionally coated, e.g. as in PCT/GB97/00067), or a vesicle, e.g. an iodinated contrast agent containing micelle or liposome.

Put briefly, for the imaging modalities of MRI, X-ray, light imaging, nuclear imaging, magnetotomography and electrical impedance tomography, the favoured reporters may be as follows:

MRI

Superparamagnetic iron oxide particles, in general having a particle size smaller than about 80 nm and especially those with a size less than 20 nm. In particular iron oxides coated with various coating materials such as polyelectrolytes, PEG, starch and hydrolyzed starch are preferred. Paramagnetic metal substances including both chelates and particulate materials are also useful.

Light imaging

Any light imaging reporter group. The focus should be on substances

absorbing in the near infrared range.

Nuclear medicine	Radioactive chelates comprising ^{99}Tc or ^{111}In as well as direct radiolabelled vectors having radiolabelled halogens substituents such as ^{123}I , ^{125}I , ^{131}I , ^{75}Br or ^{77}Br .
Magnetotomography	Superparamagnetic iron oxide particles as described above.
Electrical impedance tomography	Polyionic species, e.g. polymers with ionic groups in the repeat units.

A preferred embodiment of the invention relates to a radiolabelled agent of general formula (I), particularly for use in tumour imaging.

The diagnostic agents of the invention may be administered to patients for imaging in amounts sufficient to yield the desired contrast with the particular imaging technique. Where the reporter is a metal, generally dosages of from 0.001 to 5.0 mmoles of chelated imaging metal ion per kilogram of patient bodyweight are effective to achieve adequate contrast enhancements. For most MRI applications preferred dosages of imaging metal ion will be in the range of from 0.02 to 1.2 mmoles/kg bodyweight while for X-ray applications dosages of from 0.05 to 2.0 mmoles/kg are generally effective to achieve X-ray attenuation. Preferred dosages for most X-ray applications are from 0.1 to 1.2 mmoles of the lanthanide or heavy metal compound/kg bodyweight. Where the reporter is a radionuclide, dosages of 0.01 to 100 mCi, preferably 0.1 to 50 mCi will normally be sufficient per 70 kg

bodyweight. Where the reporter is a superparamagnetic particle, the dosage will normally be 0.5 to 30 mg Fe/kg bodyweight.

The dosage of the compounds of the invention for therapeutic use will depend upon the condition being treated, but in general will be of the order of from 1 pmol/kg to 1 mmol/kg bodyweight.

The compounds according to the invention may be formulated for administration using physiologically acceptable carriers or excipients in a manner fully within the skill of the art. For example, the compounds, optionally with the addition of pharmaceutically acceptable excipients, may be suspended or dissolved in an aqueous medium, with the resulting solution or suspension then being sterilized.

The agents of formula I may be therapeutically effective in the treatment of disease states as well as detectable in *in vivo* imaging. Thus for example the vector of the VLR compound may have therapeutic efficacy, e.g. by virtue of the radiotherapeutic effect of a radionuclide reporter, the efficacy in photodynamic therapy of a chromophore (or fluorophore) reporter or the chemotherapeutic effect of the vector moiety.

Use of the agents of formula I in the manufacture of therapeutic compositions and in methods of therapeutic or prophylactic treatment of the human or non-human animal body are thus considered to represent further aspects of the invention.

Viewed from a further aspect the invention provides the use of an agent of formula I for the manufacture of a contrast medium for use in a method of diagnosis

involving administration of said contrast medium to an animate subject and generation of an image of at least part of said subject.

Viewed from a still further aspect the invention provides a method of generating an image of an animate human or non-human (preferably mammalian or avian) animal subject involving administering a contrast agent to said subject, e.g. into the vascular system and generating an image of at least a part of said subject to which said contrast agent has distributed, e.g. by X-ray, MR, ultrasound, scintigraphy, PET, SPECT, electrical impedance, light or magnetometric imaging modalities, characterised in that as said contrast agent is used an agent of formula I.

Viewed from a further aspect the invention provides a method of monitoring the effect of treatment of a human or non-human animal subject with a drug to combat a condition associated with angiogenesis, e.g. a cytotoxic agent, said method involving administering to said subject an agent of formula I and detecting the uptake of said agent by endothelial cell receptors, in particular $\alpha v \beta 3$ receptors, said administration and detection optionally but preferably being effected repeatedly, e.g. before, during and after treatment with said drug.

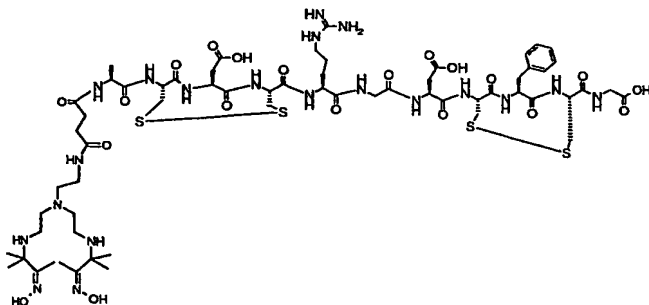
Viewed from a yet further aspect the invention provides a process for the preparation of an agent of formula I, said process comprising the conjugation of a vector V to a compound detectable in a diagnostic imaging procedure or a chelant compound and if necessary metallating chelant groups in the resultant conjugate with a metal ion detectable in a diagnostic imaging procedure.

Viewed from yet another aspect the invention provides a process for the preparation of an agent of formula I, for therapeutic treatment, said process comprising the conjugation of a vector V to a compound to be therapeutically effective in the treatment of disease states.

The vectors of the present invention can be synthesised using all the known methods of chemical synthesis but particularly useful is the solid-phase methodology of Merrifield employing an automated peptide synthesiser (J. Am. Chem. Soc., 85: 2149 (1964)). Vectors containing multiple disulphide bridges are synthesised using differential cysteine protecting groups so that no ambiguity exists as to the final folded form of the vector. The peptides and peptide chelates may be purified using high performance liquid chromatography (HPLC) and characterised by mass spectrometry and analytical HPLC before testing in the in vitro screen.

There follows a series of non-limitive examples:

Example 1: Synthesis of compound 1:



Synthesis of technetium chelate - Pn216

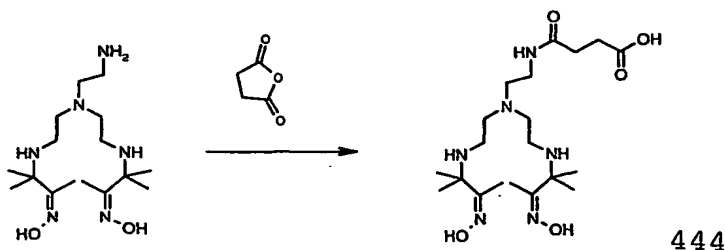
a) Chloro-nitroso intermediate (3-chloro-3-methyl-2-nitrosobutane)

A mixture of 2-methylbut-2-ene (18.5 mL) and iso-amyl nitrate (19.5 mL) was stirred, cooled to -10 °C and concentrated hydrochloric acid (17.5 mL) added carefully to maintain the temperature below 0 °C. The reaction was stirred at this temperature for 30 minutes. The precipitate formed was collected by filtration, washed 4 X 5 mL of ethanol (-20 °C) and dried in vacuo to give 3-chloro-3-methyl-2-nitrosobutane as a white solid.

b) Pn216 - (3,3,11,11-tetramethyl-7-aminoethyl-4,7,10, triazatridecane-2,12-dionedioxime)

To a solution of tris-(2-aminoethyl) amine in acetonitrile (20 mL) was added sodium bicarbonate (2.2g, 26 mmol). A solution of 3-chloro-3-methyl-2-nitrosobutane (1.8g, 13 mmol) in dry acetonitrile was added slowly at 0 °C. The reaction mixture was left to stir at room temperature for 4 hours and then filtered. The filtrant was washed with acetonitrile and the filtrate evaporated. The crude product was dissolved in acetonitrile and purified by HPLC to afford Pn216. Yield 0.88g, 19%

c) Synthesis of Pn216-succinic acid intermediate:



Succinic anhydride (100)

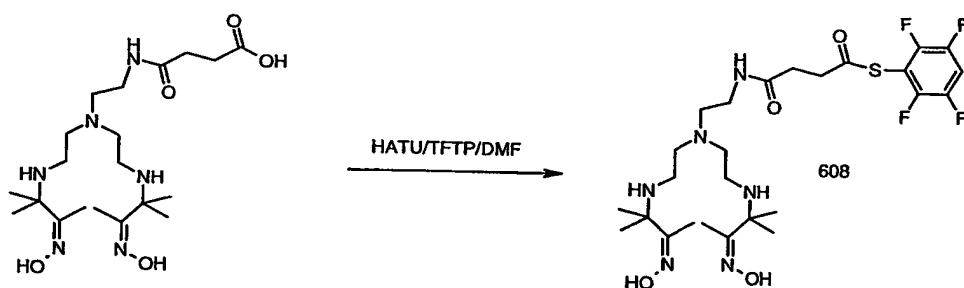
Pn216 (358)

Tetrafluorothiophenol (182)

DCCI (206)

Pn216 (0.5g, 1.4 mmol) was dissolved in DMF (5 mL) and succinic anhydride (0.015g, 1.5 mmol) in DMF (10mL) added portionwise with stirring. The reaction was left stirring for 16 hours to afford complete conversion to the desired product. The pure acid was obtained following HPLC chromatography in good yield.

d) Synthesis of the tetrafluorothiophenol ester derivative of Pn216-succinic acid



HATU [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] - Mwt=380

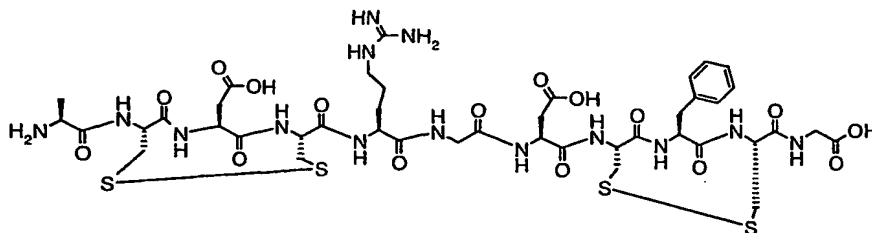
Pn216-NH-CO-(CH₂)₂-COOH - Mwt= 458

NMM - N-methylmorpholine - Mwt = 101

TFTP - tetrafluorothiophenol - Mwt = 182

To Pn216 acid (10 mg, 0.022 mmol) in DMF (1.0 mL) was added HATU (8.3 mg, 0.022 mmol) and NMM (0.007 mL, 0.066mmol). The mixture was stirred for 5 minutes then TFTP (0.022 mmol, 4 mg) added. The solution was stirred for 30 minutes then the reaction mixture was diluted with 20% acetonitrile/ H₂O (3 mL) and the product purified by reverse-phase chromatography yielding 6 mg of the desired product following freeze-drying.

e) Synthesis of peptide vector NH₂-Ala-Cys-Asp-Cys-Arg-Gly-Asp-Cys-Phe-Cys-Gly-OH with disulphide bonds connecting Cys 2 and 4 ; Cys 8 and 10



The peptide was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Gly Wang resin (Novabiochem) on a 0.1 mmol scale using 1 mmol amino acid cartridges. Cysteine residues 2 and 4 were S-protected using trityl protection while 8 and 10 were protected with acetamidomethyl (Acm) protection. The amino acids were pre-activated using HBTU before coupling. The simultaneous removal of peptide and side-chain protecting groups (except Acm) from the resin was carried out in TFA containing TIS (5 %), H₂O (5 %) and phenol (2.5 %) for two hours.

After work-up 100 mg of partially protected crude peptide was obtained (Analytical HPLC: Gradient, 0-30 % B over 20 min where A = H₂O/0.1 % TFA and B = CH₃CN/0.1 % TFA; column, VYDAC C18 218TP54; detection, UV 214 nm; product retention time, 16.7 min). An aliquot of crude product (25 mg) was then purified by Prep. HPLC Vydac column yielding 12.5 mg of pure partially protected peptide.

The first disulphide bond between Cys2 and Cys4 was formed by dissolving the pure intermediate in 20 mL of 2.5% DMSO/TFA solution. After 40 minutes a new peak had appeared corresponding to the oxidised product. To the peptide solution was then added anisole (0,02 mL) and the solution warmed to 60 °C for 50 minutes. Excess TFA was then removed in vacuo and the product precipitated following addition of diethyl ether. A further Prep. HPLC step was carried out and pure product collected and freeze-dried. MALDI-TOF analysis was used

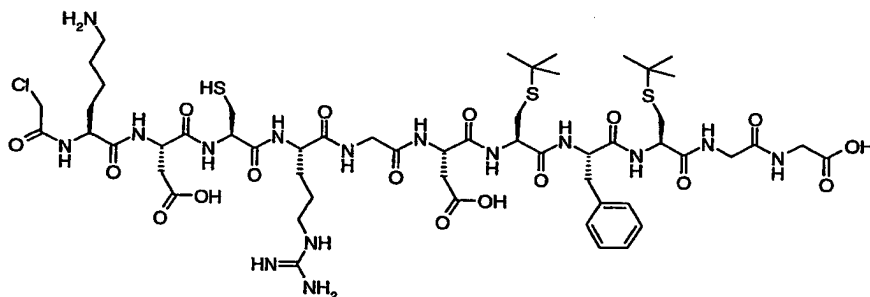
to confirm molecular weight and a co-injection with the two other possible disulphide isomers performed to confirm identity.

f) Synthesis of compound 1:

Peptide from section e) above is dissolved together with Pn216 active ester from section d) above in DMF in a 1:2 ratio (w:w). The reaction is left stirring for 2 days then the mixture is diluted with water and the desired product purified out by reverse-phase HPLC.

Example 2: [Cys^{2-4, 8-10}] analogues

a) Synthesis of ClCH₂CO-Lys-Asp-Cys-Arg-Gly-Asp-Cys(tBu)-Phe-Cys(tBu)-Gly-Gly-OH



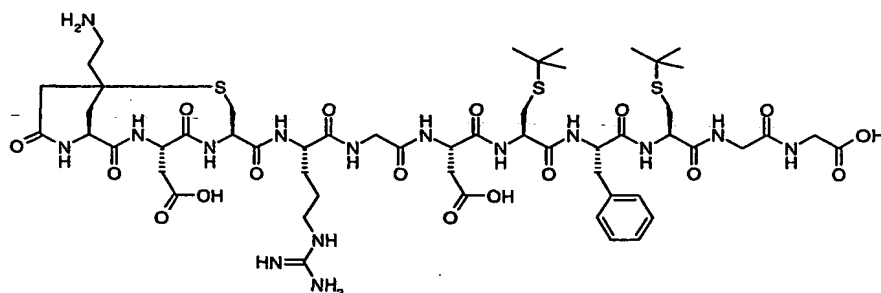
The peptide was synthesised on an ABI 433A automatic peptide synthesiser starting with Fmoc-Gly-Wang resin on a 0.25 mmol scale using 1 mmol amino acid cartridges. The amino acids were pre-activated using HBTU before coupling. Final N-terminal chloroacetylation was accomplished using a solution of chloroacetic anhydride in DMF for 30 min.

The simultaneous removal of peptide and side-chain protecting groups (except tBu) from the resin was carried out in TFA containing TIS (5 %), H₂O (5 %) and phenol (2.5 %) for two hours.

After work-up 260 mg of crude peptide was obtained (Analytical HPLC: Gradient, 5-50 % B over 10 min where A = H₂O/0.1 % TFA and B = CH₃CN/0.1 % TFA; column,

Phenomenex Luna 3 μ C18 (2) 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 6.5 min. Further product characterisation was carried out using mass spectrometry: Expected, M+H at 1348.5, found, at 1348.5).

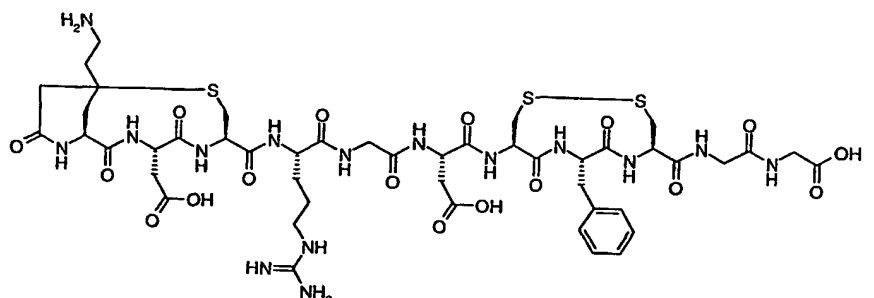
b) Synthesis of cyclo[CH₂CO-Lys-Asp-Cys]-Arg-Gly-Asp-Cys(tBu)-Phe-Cys(tBu)-Gly-Gly-OH



100 mg ClCH₂CO-Lys-Asp-Cys-Arg-Gly-Asp-Cys(tBu)-Phe-Cys(tBu)-Gly-Gly-OH was dissolved in water/acetonitrile. The mixture was adjusted to pH 8 with ammonia solution and stirred for 24 hours.

After work-up crude peptide was obtained (Analytical HPLC: Gradient, 5-50 % B over 10 min where A = H₂O/0.1 % TFA and B = CH₃CN/0.1 % TFA; column, Phenomenex Luna 3 μ C18 (2) 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 6.32 min. Further product characterisation was carried out using mass spectrometry: Expected, M+H at 1312.5, found, at 1312.6).

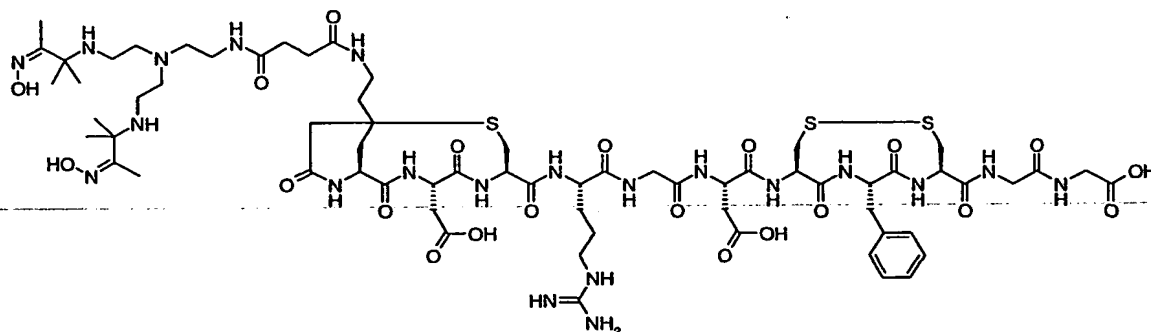
c) Synthesis of [Cys⁷⁻⁹] cyclo[CH₂CO-Lys-Asp-Cys]-Arg-Gly-Asp-Cys-Phe-Cys-Gly-Gly-OH



40 mg of cyclo[CH₂CO-Lys-Asp-Cys]-Arg-Gly-Asp-Cys(tBu)-Phe-Cys(tBu)-Gly-Gly-OH was treated with a solution of anisole (200 μ L), DMSO (1 mL) and TFA (50 mL) for 30 min following which the TFA was removed *in vacuo* and the peptide precipitated by the addition of diethyl ether.

Purification by preparative HPLC (Phenomenex Luna 5 μ C18 (2) 250 x 21.20 mm column) of the crude material (40 mg) was carried out using 0-30 % B, where A = H₂O/0.1 % TFA and B = CH₃CN/0.1 % TFA, over 40 min at a flow rate of 10 mL/min. After lyophilisation 14.3 mg of pure material was obtained (Analytical HPLC: Gradient, 0-30 % B over 10 min where A = H₂O/0.1 % TFA and B = CH₃CN/0.1 % TFA; column, Phenomenex Luna 3 μ C18 (2) 50 x 4.6 mm; flow, 2 mL/min; detection, UV 214 nm; product retention time, 6.10 min. Further product characterisation was carried out using mass spectrometry: Expected, M+H at 1198.4, found, at 1198.5).

d) Conjugation of [Cys⁷⁻⁹] cyclo[CH₂CO-Lys-Asp-Cys]-Arg-Gly-Asp-Cys-Phe-Cys-Gly-Gly-OH and Pn216-succinic acid

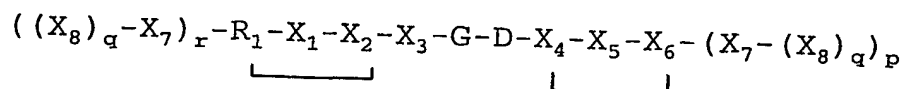


[Cys⁷⁻⁹] cyclo[CH₂CO-Lys-Asp-Cys]-Arg-Gly-Asp-Cys-Phe-Cys-Gly-Gly-OH, Pn216 chelate active ester and N-methylmorpholine is dissolved in DMF. The mixture is stirred until complete conjugation is observed by RP-HPLC monitoring.

Purification by preparative RP-HPLC of the reaction mixture is carried out to afford pure material following lyophilisation. Product characterisation is carried out using RP-HPLC and mass spectrometry.

Claims:

1. A compound of general formula (I)



(I)

wherein

$r = 0$ or 1

$p = 0$ or 1

and $r + p = 1$

when $r = 0$ then R_1 is $-(CH_2)_n-CO-$ or $-(CH_2)_n-C_6H_4-CO-$
where $n=1,2,3,4$ or 5 ,

when $r = 1$ then R_1 is or one or more bridge-forming amino acids,

X_1 = a bond or 1, 2, 3, 4 or 5 amino acids, or an amino acid derivatized with a carbohydrate moiety, or an amino acid functionalised with a spacer or linker and/or a chelate binding or capable of binding a reporter suitable for in vivo imaging, ,

X_2 and X_4 are independently cysteine, homocysteine or other amino acids capable of forming a cyclising bond such as aspartic acid and lysine,

X_3 is arginine, N-methylarginine or an arginine mimetic,

X_5 is a hydrophobic amino acid,

X_6 is an amino acid capable of forming a cyclising bond,

X_7 is a bond or 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids, or a spacer or linker, optionally allowing for labelling with multiple chelates as defined by X_8 , and

optionally comprising one or more ethylene glycol units or any other spacer component, and

X_6 is a chelate binding to, or capable of binding a metal radionuclide or any other reporter suitable for in vivo imaging, or is $-NH_2$ or is absent,

q is 0, 1, 2, 3, 4, 5, 6, 7 or 8, and

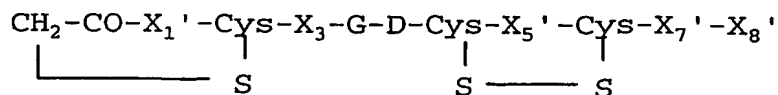
one of the bridges (between R_1 and X_2 or between X_4 and X_6) comprises a disulphide bond.

2. A compound as claimed in claim 1 wherein R_1 is cysteine.

3. A compound as claimed in claim 1 wherein X_6 is cysteine or homocysteine.

4. A compound as claimed in claim 1 wherein X_6 is phenylalanine, tyrosine, iodotyrosine, diiodotyrosine or naphthylalanine.

5. A compound as claimed in claim 1 and defined by formula II



(II) where Cys= Cysteine

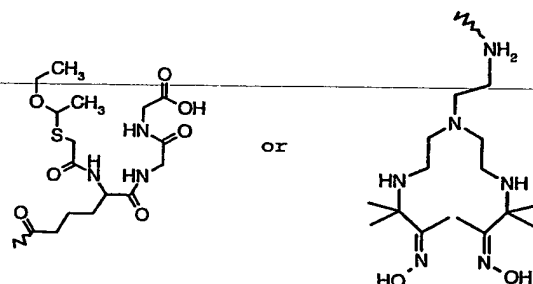
wherein

X_1' is 1, 2 or 3 amino-acids, most preferably aspartic acid, tyrosine, tyrosine-aspartic acid, lysine or acetyl-lysine, or mixtures thereof;

X_2 is as defined in formula I;

X_6' is phenylalanine, tyrosine, 3-iodo-tyrosine or naphthylalanine;

X_7' is a bond, glycine, or a O-bis(aminoethyl)ethylene glycol spacer, preferably X_7 is glycine; and X_8' is a chelate binding to a metal radionuclide wherein the structure of the chelate is



or any other N_3S or bis-oxime type of these chelates, and

one bridge comprises a thio bond and the other bridge comprises a disulphide bond as shown in formula II.

6. A compound as claimed in claim 5 wherein X_1' is aspartic acid, tyrosine, tyrosine-aspartic acid, lysine or acetyl-lysine

7. A compound as claimed in claim 5 wherein X_7' is glycine.

8. A pharmaceutical composition comprising an effective amount of a compound of general formula (I) or an acid addition salt thereof to enhance image contrast for *in vivo* imaging or for therapeutic treatment, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

9. A method of generating enhanced images of a human or non-human animal body which comprises administering to said body a pharmaceutical composition as claimed in claim 8 and generating an image of at least a part of said body.

10. A method of monitoring the effect of treatment of a human or non-human animal subject with a drug to combat a condition associated with angiogenesis which comprises administering to said subject a compound of formula I and detecting the uptake of said compound, said administration and detection optionally but preferably being effected repeatedly, e.g. before, during and after treatment with said drug.

11. Use of a compound of formula I in the manufacture of therapeutic compositions and in methods of therapeutic or prophylactic treatment of the human or non-human animal body and/or for the manufacture of a contrast medium for use in a method of diagnosis involving administration of said contrast medium to an animate subject and generation of an image of at least part of said subject.

12. A process for the preparation of a compound of formula I, said process comprising the conjugation of a vector V to a compound detectable in a diagnostic imaging procedure or a chelant compound and if necessary metallating chelant groups in the resultant conjugate with a metal ion detectable in a diagnostic imaging procedure.

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A61K 38/08, 51/00

Alan [GB/NO]; Amersham Health AS, Nycoveien 1-2,
P.O. Box 4220 Nydalen, N-N-0401 Oslo (NO).

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(26) Publication Language: English

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Filed on 5 January 2001 (05.01.2001)

(71) Applicant (for all designated States except US): **AMER-
SHAM HEALTH AS** [NO/NO]; Intellectual Property
Dept., Nycoveien 1-2, P.O. Box 4220 Nydalen, N-N-0401
Oslo (NO).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **CUTHBERTSON,**

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
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GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
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— with international search report

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For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: PEPTIDE-BASED COMPOUNDS

(57) Abstract: This invention relates to new peptide-based compounds and their use in therapeutically effective treatments as well as for diagnostic imaging techniques. More specifically the invention relates to the use of such peptide-based compounds used as targeting vectors that bind to receptors associated with angiogenesis, in particular the avb3 integrin receptor. Such contrast agents may thus be used for diagnosis of for example malignant diseases, heart diseases, inflammation-related diseases, rheumatoid arthritis and Kaposi's sarcoma. Moreover such compounds may also be used in therapeutic treatment of these diseases.

WO 02/026776 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 01/00390

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 7/06, A61K 38/08, A61K 51/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEM.ABS.DATA, WPI DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9514714 A1 (LA JOLLA CANCER RESEARCH FOUNDATION), 1 June 1995 (01.06.95) --	1-12
A	WO 9015818 A1 (ANTISOMA LIMITED), 27 December 1990 (27.12.90) -- -----	1-12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 March 2002

Date of mailing of the international search report

15. 04. 2002

Name and mailing address of the ISA/
European Patent Office

Facsimile No.

Authorized officer

Carolina Gómez Lagerlöf/BS

Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO01/00390**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9-11
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO01/00390

Claims 9-11 relate to methods of treatment of the human or animal body by surgery or by therapy/ diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/01/02

International application No.

PCT/NO 01/00390

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9514714	A1	01/06/95	AU	682561 B	09/10/97
				AU	1259695 A	13/06/95
				DE	69427370 D	00/00/00
				EP	0730607 A,B	11/09/96
				EP	0906919 A	07/04/99
				JP	9509142 T	16/09/97
				US	5627263 A	06/05/97
				US	5981478 A	09/11/99

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				DE	69026685 D,T	24/10/96
				DK	429626 T	12/08/96
				EP	0429626 A,B	05/06/91
				ES	2087155 T	16/07/96
				GB	2241243 A,B	28/08/91
				GB	8914020 D	00/00/00
				GB	9103416 D	00/00/00
				JP	2740794 B	15/04/98
				JP	4505330 T	17/09/92
				US	5395609 A	07/03/95
				US	5843402 A	01/12/98
				US	6217846 B	17/04/01

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- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

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